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Infection of Five Human Liver Cell Lines by Dengue-2 Virus

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Elevated serum transaminase levels of dengue patients indicate the possible impact of dengue virus infection on liver function. To elucidate the action of dengue virus infection in liver cells, an in vitro cell line system was established that mimicked the liver status of diverse clinical patients. Briefly, four hepatoma cell lines (HA22T, Huh7, Hep3B, and PLC) and one nonmalignant hepatocyte cell line (Chang liver) were included, representing various levels of tumorigenicity and differentiation. Our data showed that in these five cell lines, dengue-2 virus attached to each cell type equally well; however, this virus had higher replication rates and levels of virion production in differentiated Huh7, PLC, Hep3B, and Chang liver cells. Likewise, a lower replication rate was observed in the de-differentiated HA22T cells. Differentiation-related factors seem to play an important role in dengue virus replication. Further study showed that sodium butyrate (NaB, a differentiation inducer) treatment enhanced dengue virus replication in HA22T cells. Moreover, we found that the severity of morphologic aberration and the increase in aspartate aminotransferase (AST) levels correlated with the virus replication rate in the four infected hepatoma cells. In conclusion, we showed that dengue virus can infect diverse liver cells with differing replication efficiency, which causes cytopathic effects (CPEs) of diverse severity. Among the CPEs, the increased AST levels correlated with the clinical results from 24 dengue fever patients, who showed increased AST levels at the onset of fever. In summary, we find that dengue-2 virus replicates actively and causes severe CPEs in differentiated hepatoma cells. Factors related to differentiation as well as tumorigenicity seem to play critical roles, though the mechanisms of action remain unclear. ***J. Med. Virol. 60:425–431, 2000.***

KEY WORDS: dengue virus; hepatoma cell lines; aspartate aminotransferase; sodium butyrate

INTRODUCTION

Dengue viruses (serotypes 1 to 4) belonging to the Flaviviridae are mosquito-born viruses. Twenty billion people worldwide are at risk of being infected each year. Patients with dengue virus infection show a wide range of clinical effects, from mild fever to life-threatening symptoms of hemorrhagic fever and/or shock syndrome (DHF/DSS) [Halstead, 1989]. Elevated aspartate aminotransferase (AST) levels have been detected in dengue virus–infected patients; moreover, the AST level in serum of DHF patients is higher than that of patients with dengue fever alone [Wang et al., 1990; Kuo et al., 1992; Kalayanarooj et al., 1997]. Dengue virus antigen has been detected in hepatocytes, and virus particle was recovered from the liver biopsy specimens of DHF patients [Burke, 1968; Rosen et al., 1989; Hall et al., 1991]. In addition, centrilobular necrosis, fatty change, Kupffer cell hyperplasia, acidophilus bodies, and monocyte infiltration of the portal tract were also detected in DHF/DSS patients [Bhamarapravati et al., 1967; Burke, 1968; Bhamarapravati, 1989]. The cited reports indicate malfunction of the liver, which may be caused by either direct dengue virus infection of liver cells or dengue virus–mediated host immune responses. Marianneau et al. [1996, 1997] showed that dengue-1 virus could replicate in the human hepatoblastoma cell line HepG2 and induce cell apoptosis. Since hemostatic abnormalities are detected in DHF/DSS patients and since the liver is the organ to synthesize the major hemostatic factors (such as coagula-

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tion factors and fibrinogen), it is important to clarify the role of dengue virus infection in liver function and its relationship to DHF/DSS.

From November 1998 to January 1999, an outbreak of dengue fever occurred in the southern part of Taiwan. The transaminase levels of 24 patients were analyzed, to evaluate whether dengue virus infection affects liver function. At the same time, five liver cell lines, showing different levels of tumorigenicity and differentiation, were used to verify the clinical observations and to assess whether these different features affect dengue-2 virus replication in these cells. Accordingly, we compared dengue-2 virus replication rates and the cytopathic effects (CPEs) in these cell lines. We also explored the possible factors involved in controlling dengue virus replication rate and CPE severity.

MATERIALS AND METHODS

Patients

Twenty-four patients (15 males and 9 females, with ages ranging from 7 to 78 years) who were hospitalized in the National Cheng Kung University Hospital showed clinical symptoms of fever, rash, and bone pain during a dengue fever outbreak from November 1998 to January 1999. Dengue-3 virus infection was confirmed by anti-dengue enzyme-linked immunosorbent assay IgM or reverse transcription–polymerase chain reaction. These patients all showed negative results for IgM to hepatitis B virus core Ag. Biochemical analysis of liver function, including AST levels (normal value < 37 U/L), and alanine aminotransferase (ALT) (normal value < 40 U/L) were performed either within 1 week of the onset of fever or 10 to 14 days after the onset of fever. These data were analyzed statistically by paired *t* test.

Cell Lines and Virus

Chang liver cell (a nonmalignant liver epithelial cell line of human origin) [Matsuguchi et al., 1990], HA22T, Huh7, Hep3B, and PLC (human hepatoma cell lines) were cultured at 37°C in a 5% CO_2 incubator. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (TRACE, Australia), 200 U/ml penicillin, and 100 μg/ml streptomycin. Hepatocytes were infected with 100 multiplication of infection (m.o.i.) of dengue-2 virus at 37°C for 2 hr. The cells were then washed three times with phosphate-buffered saline (PBS) and incubated at 37°C with DMEM containing 10% FBS. Dengue-2 virus (PL046 Taiwan isolated) was maintained in C6/36 cells. To prevent selection of mutants, the virus was passaged no more than three times in these cells. The titration was evaluated in BHK-21 cells by plaque assay. The dengue-2 virus titer used for all the experiments was 100 m.o.i..

Plaque Assay

BHK-21 cells (1×10^5/well) were plated onto 24-well plates overnight and then infected with 200 μl of serially diluted virus solution. After adsorption for 2 hr, the virus solution was replaced with DMEM containing 2% FBS and 1% methylcellulose (Sigma, St. Louis, MO). On the fifth day after infection, the methylcellulose solution was removed, and the cells were fixed and stained with crystal violet solution (1% crystal violet, 0.64% NaCl, 2% formalin).

RNA Extraction

Total cellular RNA was extracted by guanidine isothiocyanate lysis buffer (4 mol/L guanidine isothiocyanate, 25 mmol/L sodium citrate, 50 mmol/L 2-mercaptoethanol, and 0.5% sarkosyl). The solution was sequentially mixed with 1-10 volume of 3 mol/L sodium acetate (pH 5.2), an equal volume of water-equilibrated phenol, and 1-5 volume of chloroform. After centrifugation at 14,000 rpm for 20 min, the aqueous phase was combined with 2.5 volume of 95% ethanol to precipitate the RNA. The resulting RNA was dissolved in diethylpyrocarbonate (Sigma) water and quantified by absorbency at optical density 260 nm [Liu et al., 1995, 1997].

Primers and Probes

The dengue-2-specific probe was synthesized by asymmetry polymerase chain reaction (PCR) [Liu et al., 1995, 1997]. Briefly, a 419-bp cDNA of dengue-2 virus was synthesized by reverse transcription–PCR using the primers of AD3 (3412-3428; 5′-CTGATTTCCATCCCGTA-3′) and AD4 (3009-3028; 5′-GATATGGGTTATTGGATGGA-3′) [Henchal et al., 1991]. The cDNA was purified for asymmetry PCR labeling in a reaction solution of 100 μmol/L dATP, dGTP, and dTTP; 10× reaction buffer (Kevin Science Technology, Brea, CA); 1 U of biothermal DNA polymerase (Kevin Science Technology); 10 μCi of [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK); 1 μmol/L of AD4 primer; and 50 ng of cDNA. The reaction was then performed in a PCR thermal cycler (Perkin-Elmer, Norwalk, CT) for 2 min at 94°C, 30 sec at 94°C, 30 sec at 50°C, 50 sec at 72°C for 30 cycles and, at the end at 72°C for 7 min [Liu et al., 1997]. A *Pst*I-digested 1,250-bp fragment of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) cDNA from plasmid pIBI30GAPDH [Fort et al., 1985] was labeled with [α-^{32}P]dCTP as the probe, using a megaprime DNA labeling system (Amersham).

Slot Blotting

Total cellular RNA (20 μg) was depolarized with 1 mol/L glyoxal and 10 mmol/L sodium phosphate at 50°C for 1 hr and then added onto the Hybond-N transfer membrane (Amersham) by a Bio-Dot SF blotter (Bio-Rad, Hercules, CA). The membrane was further cross-linked by ultraviolet light and hybridized with 2×10^6 cpm/ml of [α-^{32}P]dCTP-labeled probes. The blot was exposed to Kodak X-OMAT AR film and then quantified by a gel scanner with densitometer function (Vilber Lourmat, France) [Liu et al., 1997].

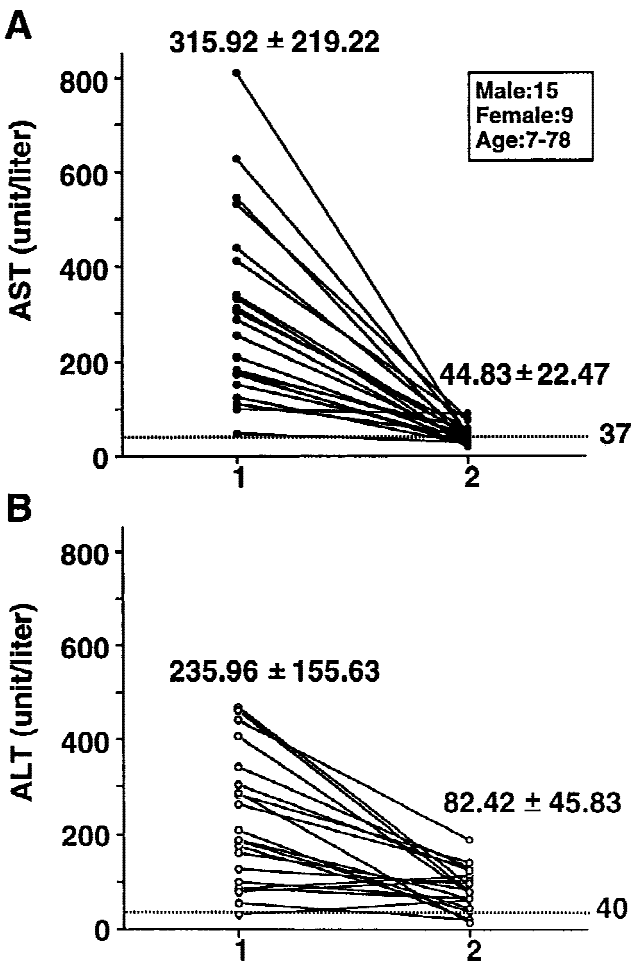

Fig. 1. The AST and ALT levels in dengue virus infected patients. Twenty-four patients (15 males and 9 females, ranging in age from 7 to 78 years) were examined for AST (**A**) and ALT (**B**) levels in serum. The data were analyzed statistically by paired *t* test. 1: At the onset of fever (within 1 week of dengue virus infection); 2: 10–14 days after the onset of fever. The dotted line represents the basal level of AST (37 U/L) and ALT (40 U/L).

Liver Function Test

Hepatocytes were cultured in 24-well plates, and the culture medium was collected for measuring AST and ALT activities using a routine automatic analyzer (Hitachi 747; Hitachi, Japan) in the clinical analysis laboratory of the National Cheng Kung University Hospital.

RESULTS

Elevated Transaminase Levels in Patients With Dengue Virus Infection

The liver function of 24 patients diagnosed with dengue fever was evaluated at the beginning of the fever and 10 to 14 days after the fever by AST and ALT analyses. The distribution of transaminase levels in serum is shown in Fig. 1. At the beginning of the fever, the AST and ALT levels were elevated to 315.92 ± 219.22 U/L and 235.96 ± 155.63 U/L, respectively. AST levels were increased more than ALT levels ($P < 0.05$). After 10 to 14 days of fever, AST levels declined to nearly normal (37 U/L), at 44.83 ± 22.47 U/L, but the ALT levels were still higher than normal (40 U/L), at 82.42 ± 45.83 U/L. In summary, the patients with dengue fever had elevated transaminase levels, indicating that dengue virus infection may affect liver function. To clarify whether dengue virus can replicate in liver cells and sequentially affect liver function, an in vitro cell culture system was established.

Dengue-2 Virus Infection and Different Replication Rates in Five Liver Cell Lines

To mimic the liver status of the patients, four hepatoma cell lines—HA22T, Huh7, Hep3B, and PLC—and one nonmalignant Chang liver cell line were used to investigate dengue virus infection of liver cells. The characteristics of these cells are listed in Table I. Briefly, HA22T cells are defined as de-differentiated cells, and the others are classified as differentiated cells. Hepatitis B virus (HBV) DNA integration was detected in HA22T, PLC, and Hep3B cell lines, and HBV surface antigen was detected in PLC and Hep3B cells. By comparison, Chang liver is a non-tumorigenic but differentiated liver cell line (Table I) [Aden et al., 1979; Knowles et al., 1980; Clementi et al., 1987, 1989; Lin et al., 1995]. These cell lines were used to assess whether tumorigenic status and differentiation levels affect dengue virus replication. Dengue-2 virus, at 100 m.o.i., was used to infect the cells (1×10^6/100-mm plate). At constant intervals, the total cellular RNA was extracted for viral negative strand (–) RNA detection, which represents virus replication in the cells [Liu et al., 1997]. The viral (–) RNA was detected by slot blotting using a sense strand (+) dengue-2 virus cDNA (nucleotide 3009–3428) as the probe (Fig. 2A).

Our slot blot data, quantified by densitometric gel scanner (Vilber Lourmat), showed that viral (–) RNA levels increased while the infection continued and reached their peak at either 36 hr or 48 hr after infection in these cell lines. However, the relative intensity of the RNA bands at 36 hr after infection varied; they were highest in Huh7 and PLC and lowest in HA22T cells (Fig. 2A,B). The housekeeping gene *GAPDH* was used for calibration to obtain equal RNA loading in each slot (data not shown). To confirm the observation of slot blotting, we measured viral numbers in the culture supernatant released from the infected cells by plaque assay. After the same treatment for slot blotting, the culture supernatants of these cells were harvested for plaque assay. The numbers of released virions rose, along with the increased infection time, in all the liver cell lines after a sharp decline at the eclipse phase of 12 hr postinfection; they reached a plateau 36 hr after infection. The rate of virus release was highest in Huh7 and PLC cells and lowest in HA22T, findings that are similar to the results of viral (–) RNA synthesis (Fig. 2B and Fig. 3). Taken together, our data showed that dengue-2 virus did infect the five liver cell lines, but replicated differently.

TABLE I. The Characteristics of the Five Liver Cell Lines

Cell line	Differentiation level[a]	HBV DNA integration (copy no.)	HBsAg expression	p53 mutation[b]
HA22T	Low	+ (3)	–	–/–
PLC/PRF5	High	+ (7)	+	Arg → Ser (249)
Hep3B	Very high	+ (2)	+	–/–
Huh7	Very high	–	–	Tyr → Cys (220)
Chang liver	Very high	–	–	+/+

HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.
[a]The degree of cell differentiation is based on the production of plasma proteins and the levels of human leukocyte antigen class I antigen induced by interferon-γ [Clementi et al., 1989].
[b]The p53 status was reported by Lin et al., 1995.

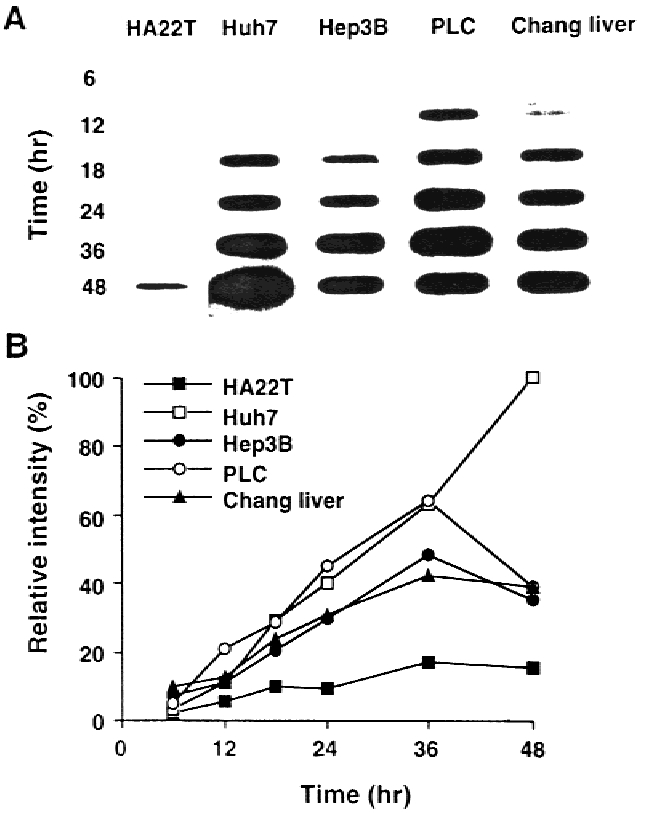

Fig. 2. Dengue-2 virus negative strand (–) RNA synthesis in the five liver cell lines was evaluated by slot blot analysis. **A:** HA22T, Huh7, Hep3B, PLC, and Chang liver cells were infected with dengue-2 virus (100 m.o.i.), and the total cellular RNA was harvested by slot blotting for viral (–) RNA detection at 6, 12, 18, 24, 36, and 48 hr after infection. A 419-bp sense cDNA of dengue-2 virus (3009–3428) was labeled with [α-^{32}P]dCTP by asymmetry PCR for the sense strand probe. **B:** The RNA band intensity in A was quantified by a densitometric gel scanner (Vilber Lourmat). The band intensity of dengue virus (–) RNA in Huh7 cells at 48 hr was set at 100%.

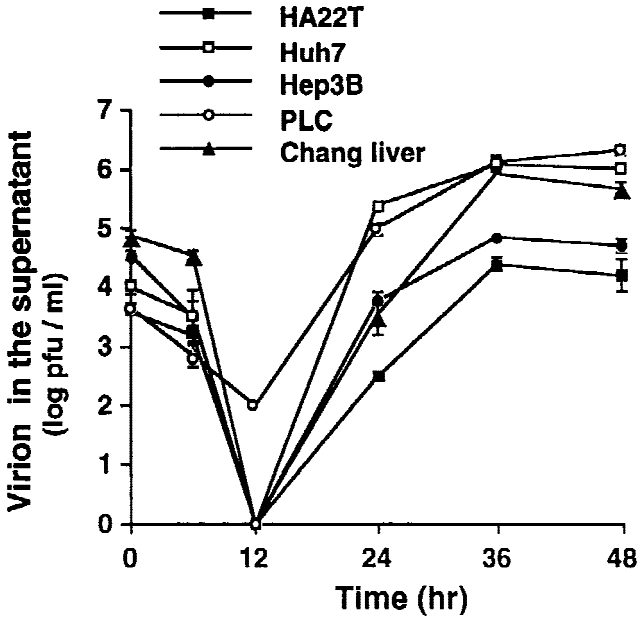

Fig. 3. Virion production in dengue-2 virus infected liver cell lines was measured by plaque assay. The supernatants of dengue-2 virus infected cells were harvested for virion detection by plaque assay at 0, 6, 12, 24, 36, and 48 hr after infection.

Various Degrees of Cytopathic Effects in the Five Liver Cell Lines Infected With Dengue-2 Virus

The dengue fever patients showed increasing levels of AST and ALT in serum (Fig. 1). Moreover, DHF patients showed much higher levels of AST and ALT, indicating malfunction of the liver [Wang et al., 1990; Kuo et al., 1992; Kalayanarooj et al., 1997]. However, whether direct dengue virus infection and/or host immune responses caused liver malfunction remains unclear. We investigated whether the increasing levels of aminotransferases were caused by direct dengue-2 virus infection of the liver cells. To test this possibility, we infected the five liver cell lines with dengue-2 virus and observed the CPEs, which include alterations of cell morphology and transaminase levels. Briefly, the infected Huh7, PLC, and Hep3B cells showed dramatic morphologic changes, including the formation of inclusion bodies in cytosol, flotation of cell debris in the culture medium, rounding of cells, and cell death. In contrast, only slight morphologic aberration was observed in HA22T and Chang liver cells (Fig. 4A).

In addition, the culture supernatants from dengue-2 virus–infected or control cells were collected for AST and ALT analysis at 24 and 48 hr after infection. The AST levels in the infected culture medium increased in an infection-time-dependent manner. However, the degree of increase varied dramatically—4.6-fold in infected Huh7 cells, 2.7-fold in PLC, 1.8-fold in Hep3B, 1.4-fold in HA22T, and 1.2-fold in Chang live cells (Fig.

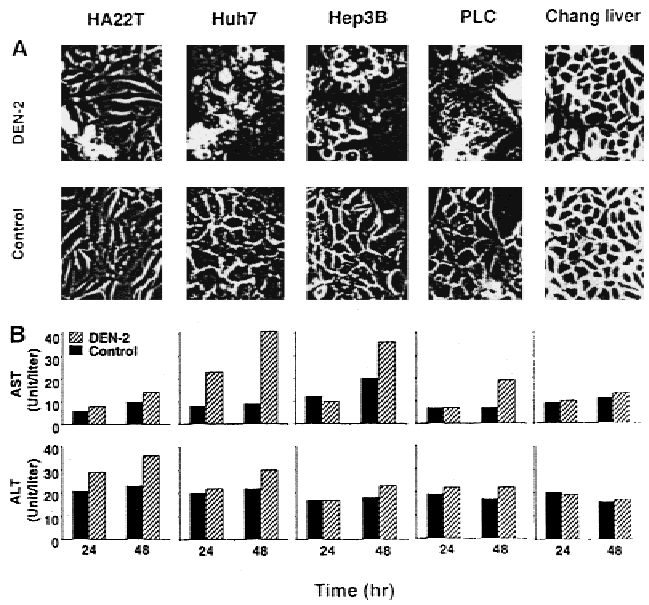

Fig. 4. Cytopathic effects of dengue-2 virus infected liver cells were evidenced by morphologic changes and increased AST and ALT levels. **A:** Morphologic changes in the five liver cell lines after dengue-2 virus infection were observed using an Olympus microscope (CK-2) at 48 hr after infection (100×). **B:** The culture supernatants of the infected cells were harvested at 24 and 48 hr after infection for AST and ALT analysis. Each data point represents the results of two repeated experiments.

4B). Evidently the increase of AST levels positively correlated with the severity of morphologic aberration of the cells (Fig. 4A,B). These phenomena also correlated with the virus replication rate in the four hepatoma cell lines. In contrast, virus replicated actively, but only mild CPEs were observed in the nonmalignant Chang liver cells. The infected hepatocytes also showed an increase of ALT levels, but they were not as high as the AST increases. There was no correlation between ALT levels and the severity of morphologic aberration (Fig. 4B).

Differentiation-related Factors Affecting Dengue-2 Virus Replication Rate in Liver Cells

To explore the possible factor(s) that might affect the virus replication rate in the cells, we first analyzed whether the adsorption of dengue-2 virus affects virus replication in the cells. The adsorption of dengue-2 virus in the five cell lines was similar, indicating that the variation in dengue-2 virus replication rate was not due to the adsorption difference between these liver cell lines (data not show).

We have shown that dengue-2 virus replicated poorly in HA22T cells (Figs. 2 and 3), defined as de-differentiated cells (as shown in Table I). It is intriguing to postulate that differentiation-related factors might affect the dengue-2 virus replication rate in the cells. It has been reported that sodium butyrate (NaB), an inhibitor of histone deacetylase, can induce differentiation in several cell types, including hepatocytes [Kaneko et al., 1990; Wadee et al., 1994; Blouin et al., 1995] and is able to activate the replication of human immunodeficiency virus, Epstein-Barr virus, herepes simplex virus, and HHV-8 [Frazier et al., 1996; Gao et al., 1997; Kashanchi et al., 1997; Kliche et al., 1998]. We used NaB to induce differentiation of HA22T cells and to evaluate whether the infectivity of dengue-2 virus could be enhanced. NaB was purchased from Sigma, and a stock solution of 1 mol/L was prepared in distilled water and stored at −20°C. HA22T cells were treated first with 2 mmol/L or 5 mmol/L of NaB for 24 hr and then infected with dengue-2 virus. NaB-treated HA22T cells showed differentiation-like morphologic characteristics and a decreased proliferation rate, indicating the induction of cell differentiation (data not shown). Subsequently, slot blot and quantitative analysis of viral (−) RNA synthesis showed that the virus replication rate indeed increased in either 2 mmol/L or 5 mmol/L of NaB treatment of HA22T cells at 36 hr post-treatment (Fig. 5A,B). The increase in virus production was also detected by plaque assay (Fig. 5C). Taken together, these findings indicate that differentiation-related factor(s) might regulate dengue virus replication in the cells.

DISCUSSION

Our clinical results showed that dengue virus–infected patients had elevated transaminase levels, indicating that dengue virus infection may affect liver function, possibly through direct destruction of liver cells and/or indirect induction of host immune responses. Our liver cell line study confirmed the direct effect of dengue virus infection on liver function, that is, dengue-2 virus replicated and caused CPEs (including an increase in AST and ALT levels) and morphologic changes in the cells. The severity of CPEs correlated with the virus replication rate in four hepatoma cell lines. On the other hand, dengue-2 virus replicated actively but caused only minor CPEs in the nonmalignant Chang liver cells (Table II). Dengue virus–infected patients showed increases of AST and ALT levels in serum. The increase of AST levels was higher than that of ALT levels at the onset of fever (Fig. 1). This phenomenon differs from the results in hepatitis virus–infected patients, in whom the ALT level is higher than that of AST [Gholson and Bacon, 1993]. The speculation is that the increased AST levels were from myocytes or monocytes [Kuo et al., 1992; Kalayanarooj et al., 1997; Nguyen et al., 1997]. Our study, however, showed that dengue virus–infected liver cells are another source of production of AST. Moreover, the half-life of ALT (32–43 hr) is longer than that of AST (12.5–22 hr) [Hawker, 1993], which explains why AST declined more rapidly to the normal level compared with ALT 10 to 14 days after fever began.

In general, both AST and ALT levels increase in dengue fever patients and dengue virus–infected liver cells, indicating that dengue virus infection affects liver function, possibly through the destruction of liver cells. Other indicators of aberrant liver function, including glucose, alkaline phosphatase, and lactate de-

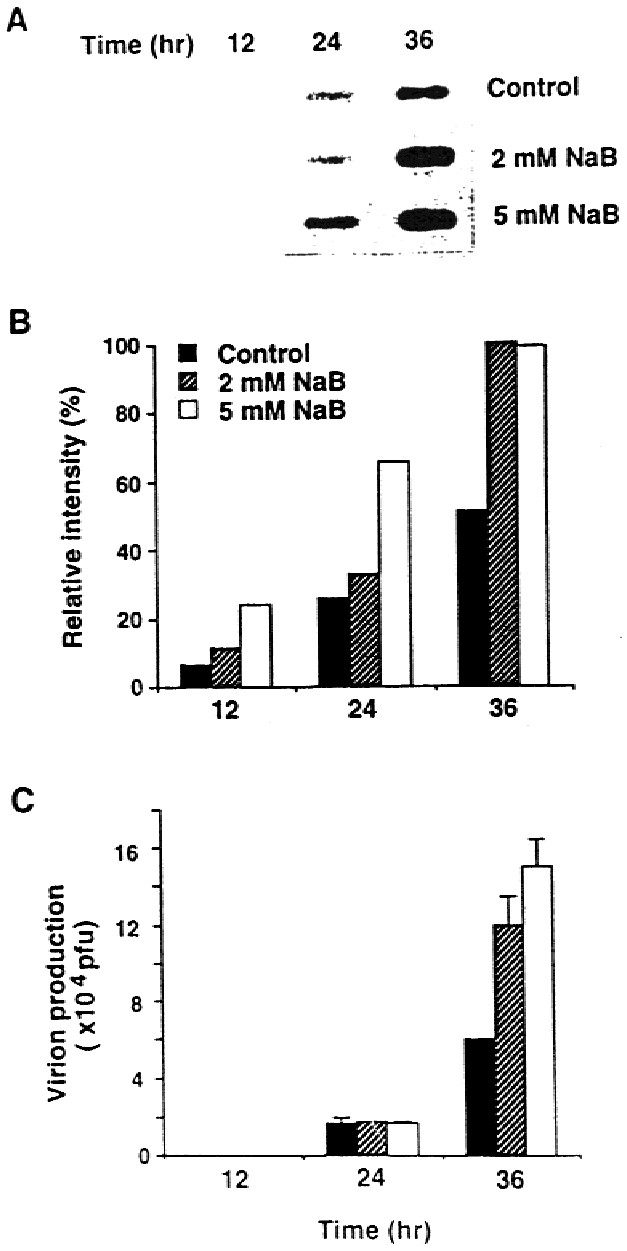

Fig 5. Dengue-2 virus replication was enhanced in HA22T cells after NaB treatment. **A:** HA22T cells were treated with 2 mmol/L or 5 mmol/L of NaB for 24 hr, followed by dengue-2 virus infection. Total RNA was extracted for viral (–) RNA detection by slot blotting at 12, 24, and 36 hr after infection. **B:** Quantified data of the RNA band intensity in A. **C:** The supernatants of infected HA22T cells were harvested for virion detection by plaque assay at 12, 24, and 36 hr after infection.

hydrogenase, were also analyzed in the five cell lines. We found that the levels of glucose and alkaline phosphatase were not affected by dengue virus infection and that the level of lactate dehydrogenase was elevated; no correlation with virus infectivity was established (data not shown). The fluctuation of AST levels is consistent with the aminotransferase levels of the dengue fever patients; thus AST could be used as one indicator for the diagnosis of dengue virus infection.

Our results, summarized in Table II, showed that the infectivity of dengue-2 virus and the severity of CPEs do not correlate with HBV DNA copy number, HBV surface antigen expression, and p53 status in the cells. Our data excluded the possibility that virus adsorption plays an important role in regulating dengue-2 virus replication in the five liver cell lines and suggested that host-specific factors play the major role in regulating replication in these cell lines. We found that dengue-2 virus infectivity correlated with the differentiation levels of the cells. Further investigation confirmed that differentiation-related host factors were indeed involved in dengue-2 virus replication, which was demonstrated by using the differentiation inducer NaB in HA22T cells. It has been reported that some HBV genes could be activated preferentially by hepatocyte nuclear factors, which would lead subsequently to HBV gene expression and virus replication in differentiated hepatoma cells [Yuh and Ting, 1993; Zhang and Mclachlan, 1994; Raney et al., 1995; Jossic et al., 1996]. Likewise, differentiation-related gene product may activate dengue virus gene expression and lead to active replication of dengue viruses. However, the exact mechanism of action and factor(s) possibly involved in the process require further investigation.

We found that the severity of CPEs, including morphologic changes and an increase of AST levels, generally correlated with virus replication rate in all the hepatoma cell lines. We speculate that certain tumor-related factors in the cells might induce dengue virus infection and cause significant CPEs. For example, alpha-fetoprotein expressed in fetus and cancer cells could transduce proliferation, differentiation, and apoptosis signals in hepatoma cells [Semenkova et al., 1997]. Apoptosis has been noted in dengue-1 virus–infected hepatoma cells [Marianneau et al., 1997]. We also detected DNA fragmentation in dengue-2 virus–infected hepatoma cells (HA22T, Huh7, Hep3B, and PLC) (data not shown). It would be interesting to iden-

TABLE II. The Relationship Between Dengue-2 Virus Infection and the Characteristics of the Five Liver Cell Lines*

	Huh7	PLC	Hep3B	Chang liver	HA22T
Differentiation level	Very high	High	Very high	Very high	Low
HBV DNA integration	No	Yes	Yes	No	Yes
Tumorigenicity	Yes	Yes	Yes	No	Yes
Dengue-2 virus replication rate	++++	++++	+++	+++	+
Aberrant morphologic features	++++	+++	++	+	+
AST level	++++	+++	++	+	+

*HBV, hepatitis B virus; AST, aspartate aminotransferase.

tify the factors affecting tumor cell activation of virus replication, leading to severe CPEs of liver cells.

From the results of this clinical investigation and our cell line study, we found that dengue virus can infect liver cells permissively, which is accompanied by increased release of AST and ALT. Moreover, our liver cell model system confirmed direct dengue-2 virus infection of diverse liver cells, contributing to the understanding of the pathogenesis of dengue viruses in the liver. In conclusion, we found that dengue-2 virus replicated actively in well-differentiated hepatocytes but poorly in de-differentiated cells. Moreover, dengue-2 virus infection caused CPEs, which correlated with the virus infection rate in these cells. We conclude from our study that dengue virus infection of the five liver cell lines produces quite different replication rates as well as CPEs, indicating that patients with dengue virus infection may show diverse forms of liver malfunction, in part dependent on the liver status of each patient. Our investigation will shed light on the pathogenesis of dengue virus infection.

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